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Original Article

Genetics Section

Single Nucleotide Polymorphism of Insulin Receptor Gene rs2059806 in Polycystic Ovary Syndrome- A Case-control Study

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ABSTRACT

Introduction: One of the most common endocrinopathies faced in clinical practice is Polycystic Ovary Syndrome (PCOS). Furthermore, it is one of the major causative factors of anovulatory infertility affecting a large number of female population worldwide. They are also at a greater risk for developing impaired glucose tolerance, type 2 diabetes mellitus and cardiovascular disease. Decreased sensitivity to insulin is a common feature observed in women with this syndrome.

Aim: To assess whether polymorphism of insulin receptor gene has a significant role to develop the PCOS.

Materials and Methods: This was a case-control, observational, hospital-based study, conducted at Department of Biochemistry, Calcutta National Medical College, Kolkata from January 2018 to September 2018. Total 123 patients with PCOS cases and 111 normo-ovulatory normal control female subjects were selected. Blood samples were collected for estimation of serum testosterone and Deoxyribonucleic Acid (DNA) extraction. Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) pattern of PCR fragments of DNA samples were determined. The study analysed and compared the genotyping and allele frequencies of rs2059806 polymorphism in control and case group. For comparison, chi-square test was performed (Odds ratio, $p<0.05$).

Results: Amongst the 123 PCOS cases and 111 normal female subjects of the study, mean age of PCOS patients were 22.59±4.7 years and that of control subjects was 21.9±5.1 years. The present study observed an increase in mutant G allele in PCOS subjects (Odds ratio-2.18, p=0.0035).

Conclusion: The polymorphism of insulin receptor gene rs2059806 may have a probable role in the development of PCOS.

Keywords: Endocrinopathies, Genetic factors, Polymerase chain reaction, Restriction fragment length polymorphism, Testosterone

INTRODUCTION

The PCOS represents a spectrum of clinical features with chronic anovulation, hyperandrogenism and ultrasound pattern of polycystic ovaries with the exclusion of other known diseases. Globally, it is considered as one of the most common gynaecological endocrinopathies [1,2]. The PCOS is a major causative factor of anovulatory infertility and the affected population has a greater risk of developing impaired glucose tolerance, type 2 diabetes mellitus, metabolic syndrome and cardiovascular diseases in later life [3]. The global prevalence of PCOS is highly variable-ranging from 2.2% to as high as 26% [4]. In India, some studies from hospital set ups and few studies among adolescents in school reports have shown the prevalence of PCOS about 9.13-36% [4-6].

Though the exact aetiology is still unclear, but various factors are involved in the disease process of PCOS, with a definite genetic predisposition. Insulin Resistance (IR), defined as the reduced cellular ability to respond to normal or elevated levels of insulin, appears to be an important pathophysiologic mechanism in the development of metabolic disturbances in PCOS [3]. Presently, IR is considered as an intrinsic component of a majority of PCOS female irrespective of being lean or obese [7].

Impaired sensitivity to insulin is a common feature observed in women with PCOS. The binding of insulin to α subunit of the Insulin receptor (INSR) gene activates the tyrosine kinase activity of the receptor triggering the signaling cascades. The post binding signaling defects of INSR have been reported in skeletal muscles and adipose tissues of women with PCOS [3]. Ranjzad F et al., didnot find any significant difference in genotype and allele frequencies between the women with PCOS and controls for rs2059806 and rs1799817, of INSR gene [8]. The observations were strengthened by the findings of Tehrani FR et al., [1].

Different population based studies were done that reported an association between C/T polymorphism of exon 17 of INSR gene and PCOS [9,10]; while others observed the association between the same polymorphism and decrease in insulin sensitivity [9,11]. Bagheri M et al., found no association between INSR gene rs1799817 and rs2059806 and PCOS in West Azerbaizan province Iran [12]. Daghestani MH observed that rs1799817 polymorphism of INSR gene was significantly associated to PCOS in lean females of Saudi Arabia [3].

Thus, the aim of the present study was to explore the rs2059806 polymorphism, exon 8 (A/G), INSR gene in PCOS female.

MATERIALS AND METHODS

This case-control, observational, hospital-based study was conducted in the Department of Biochemistry, Calcutta National Medical College, Kolkata in the period between January 2018 to September 2018. The study was approved by Institutional Ethics Committee (Memo no: CNMC/Biochem/151/2017).

Inclusion criteria: A total of 123 patients diagnosed with PCOS, with history of missed or irregular menstruation and/or infertility attending the Endocrine Outpatient Department (OPD) of the study hospital were included as cases.

The 111 age matched non hirsute normo-ovulatory female subjects selected from the female healthcare workers of the institute who were willing to participate in the study were included in the study as control subjects.

All the PCOS patients (case group) were included following Rotterdam criteria [13] (presence of two of the following: oligo/anovulation, clinical and biochemical signs of hyperandrogenism and polycystic ovaries on ultrasonography).

a. Ovaries of two sides were examined in Ultra Sonography (USG) for the following findings for the diagnosis of PCOS [14].

b. One/two ovaries demonstrating 12 or more follicles measuring 2-9 mm in diameter[3].

c. Only one ovary meeting the criteria is sufficient to establish the diagnosis of PCOS.

d. Ultrasound scanning was done in early follicular phase (day 3-5).

Exclusion criteria: Those females who were taking oral contraceptive pills, oral steroids, had a habit of drinking alcohol or smoking, underwent hormone replacement therapy, or any medications that affect endocrine parameters or lipid profile. Pregnant females and those suffering from hypertension, diabetes mellitus, dyslipidemia, thyroid diseases, hyperprolactenaemia, ovarian tumour (blood tests and USG were done for all cases and control subjects), critically ill patients and those with BMI >25 kg/m^2 were also excluded.

The machine used was GE Logiq E. Lower abdominal USG was done (transvaginal/lower abdominal route) for all cases and control subjects and following parameters in the ovary were examined: presence, number, location, size, and character of the cyst along with ovarian volume. Ovarian tumours were excluded.

Sample Collection and Handling

Fasting blood samples were collected from patients. Blood (5 mL) was withdrawn and distributed into anticoagulant-free plain tube (2 mL), and Ethylene Diamine Tetra-acetic Acid (EDTA) tube (3 mL). The blood sample in the plain tube was centrifuged after 30 minutes of sampling and serum was isolated and stored at -20°C and sent to the laboratory for biochemical analysis. Sample in the plain tube was for (S) testosterone assay. The EDTA blood was stored properly at -20°C for DNA extraction. Serum Testosterone was estimated by competitive enzyme immunoassay (Calbiotech, India) [15].

Immunoassays were measured by Automated ELISA reader (Tecan). Same samples were assayed twice and the mean of the paired results was determined.

Molecular Analysis

DNA extraction: The venous blood, which was collected in the evacuated EDTA tubes, was used for DNA extraction. The DNA from study subjects was isolated from peripheral blood (EDTA sample) using the standard phenol-chloroform method [16]. The integrity of genomic DNA was tested by resolving DNA extracts on a 0.8% agarose gel by electrophoresis (Low Electroendosmosis (EEO), Sisco Research Laboratories (SRL)), followed by visualisation with ethidium bromide staining. A quantitative spectrophotometric assay of DNA was performed with a MS-Ultraviolet (UV)+ spectrophotometer (Motras Scientific, New Delhi, India). Absorbance was measured at wavelengths of 260 and 280 (A260 and A280, respectively) nm. The absorbance quotient (Optical Density (OD)260/OD280) provides an estimate of DNA purity. An absorbance quotient value of 1.8 < ratio (R) <2.0 was considered to be good, purified DNA. The good samples were then transferred to a fresh tube, and 1 µL of 10 µg/mL RNase A (Chromus Biotech) was added. The sample was incubated at 37°C for one hour to improve the purity. The samples were stored at -20°C until use.

PCR/RFLP: Reference sequence and details of SNPs, PCR primers' design and restriction enzymes were obtained by searching the University of California Santa Cruz (UCSC) Genome Bioinformatics Site, Primer3 program and New England Biolabs (NEB) cutter program, respectively [17-20].

The genomic DNA was amplified using the following steps: Denaturation of double stranded genomic DNA at 94°C for 5 minutes, DNA amplification using 30 cycles. Each cycle consisted of: denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 40 sec, final elongation at 72°C for 7 minutes, and ending reaction at 4°C. For SNP rs2059806, the PCR primers, F: 5`CGGTCTTGTAAGGGTAACTG3`R: 5'GAATTCACATTCCCAAGACA '3, the PCR product is 325bp (Size of amplified fragment), sizes of the digested fragments of the examined SNPs were A allele 325bp and G allele 239bp and 85bp.

The restriction enzyme NsiI digestion was carried out in 1 µL containing 10 units of enzyme with 5 µL of 10X buffer and 1 µg of PCR product and incubated at 37°C for 30 minutes. Restriction enzyme was obtained from New England (BioLabs). Size of restricted fragments for A allele was 325bp and G allele was 239bp and 85bp. PCR products and digested fragments were detected using electrophoresis on 1.2% and 2% agarose, respectively.

STATISTICAL ANALYSIS

Data was analysed using the Statistical Package for Social Sciences (SPSS) version 17.0 and graph-pad software. Continuous variables were expressed as mean (standard deviation) and the differences were accomplished by comparison via unpaired two-sided t-test or one-way Analysis of variance (ANOVA) as appropriate. Discrete variables were expressed as counts and frequencies and were compared using the chi-square test. If N<5, exact Fisher statistic was used. The genotype and allele frequencies of examined Single Nucleotide Polymorphisms (SNPs) (for rs2059806) were determined (along with Odd's ratio). The genotype distributions of SNPs were analysed in agreement with the Hardy-Weinberg equilibrium. A significant difference was considered at p<0.05.

RESULTS

This study included 123 cases and 111 control subjects and their data was tabulated and analysed. [Table/Fig-1] shows that the mean testosterone levels were higher among the cases but the difference was not significant (p=0.80). The present study observed that [Table/Fig-2] there was a definite declining trend of AA genotypes in PCOS patients whereas the opposite was found in case of AG and GG genotypes. As regards allele frequency, A allele was significantly decreased whereas G allele was significantly increased in PCOS patients. Ultrasonography findings of PCOS subjects have shown that in a single section of ovary ≥10-12 follicles were present with a diameter of 2-9 mm and ovarian volume was ≥10 cm^3. The follicles were arranged throughout the ovarian stroma.

Variables studied	PCOS (n=123) mean±SD	Non PCOS control (n=111) mean±SD	p-value (unpaired t-test)
Age (years)	22.59±4.7	21.9±5.1	0.1912
Testosterone (ng/mL)	1.03	0.5	0.80

[Table/Fig-1]: Demographic, biochemical and hormonal characteristics of the studied groups.
PCOS: Polycystic ovarian syndrome

Genotypes of SNP INSR rs2059806 gene	Control n (%) (N=111)	PCOS n (%) (N=123)	Odds ratio	95%CI	p-value (chi-square test)
A/A	60 (54.05%)	43 (34.81%)	0.51	0.29-0.89	**0.01**
A/G	43 (38.73%)	60 (48.38%)	1.50	0.89-2.53	0.12
G/G	8 (7.02%)	20 (16.81%)	2.50	1.05-5.93	**0.04**
A Allele	103 (92.79%)	103 (83.73%)	0.40	0.16-0.94	**0.04**
G Allele	51 (45.94%)	80 (65.04%)	2.18	1.29-3.70	**0.0035**

[Table/Fig-2]: INSR polymorphism genotype and allele frequencies in the groups.
INSR: Insulin receptor; PCOS: Polycystic ovarian syndrome; SNP: Single nucleotide polymorphism; INSR: Insulin receptor; *Bold p-values are significant

DISCUSSION

A series of studies have been conducted to point out the relationship between INSR gene polymorphism and PCOS with diverse results [21,22]. One study conducted on rs1799817 SNP of INSR gene [3].

Insulin receptor contains α and β subunits which are encoded by exon 1-11 and exon 12-22 [23]. The INSR SNP rs1799817 is located in exon 17, within the tyrosine kinase domain. Mutations

in this restriction site can cause severe IR and hyperinsulinaemia [24]. However, it was considered to be involved in IR and PCOS particularly in lean women in Chinese and Indian, and Japanese populations. Except for the Japanese study, the frequency of polymorphic (CT+TT) genotype was higher in lean PCOS subjects [7,20,25]. In contrast, Feng C et al., didnot find any association in the same restriction site polymorphism in PCOS females [26]. Some researchers focused on rs2059806 SNP of INSR gene with diverse results. Du J et al., found very weak association between the same polymorphism and PCOS patients whereas Goodarzi MO et al., observed strong associations between them [27,28].

The present study focused on rs2059806 SNP of INSR gene (exon 8, A/G). The results clearly show that there is a significant difference in genotype and allele frequencies of INSR rs2059806 between PCOS and controls.

A meta-analysis was done by Feng C et al., on rs2059806 of INSR gene [26]. They considered three studies, one each from UK, Korea and Iran. The sensitivity analysis showed the following results-GG vs GA+AA, Odds ratio 1.57 and p=0.04. This meta-analysis showed marginally significant p-value indicating the association of rs2059806 polymorphism and PCOS patients. Tehrani FR et al., and Bagheri M et al., also studied on the same restriction site of INSR gene among Iranian population, but they didnot find significant associations [1,12].

Different population-based studies have shown diverse results on the same restriction site of INSR gene. It is believed that multiple gene such as fibrillin 3, fat and obesity associated gene, insulin receptor, insulin receptor substrate 1, may contribute to the development of PCOS. Genetic susceptibility may vary in different populations. Many other factors may be responsible for these diverse results on different populations. Body Mass Index (BMI) and ethnicity may play a significant role. Some authors didn't clearly mention the ethnic group they have studied, similarly cut-off values for lean and obese was somewhat different among different studies. Furthermore, the studies used various control groups like healthy women or infertile women which may affect the results [26].

Some studies have shown that C/T polymorphisms of INSR gene were associated with PCOS patients and impaired insulin sensitivity [9-11]. Linkage-based studies have identified D19S884, a microsatellite marker on chromosome 19 p13.2 lying in close vicinity to INSR gene having strong association with PCOS [29]. This was further confirmed in a case control study by Tucci S et al., among Caucasian women and also by Xie GB et al., among Han Chinese population [30,31]. A novel polymorphism INSR rs176477 C/T in exon 17 was identified to be associated with PCOS in Korean population [32]. Goodarzi MO et al., using tag-SNP approach, observed the association of four novel SNPs with PCOS in Caucasians [28].

While many significant SNPs are distributed in different exons and flanking regions of INSR gene, five SNPs involved in the susceptibility of PCOS concentrate in exon 9 and 5' intron of exon 9, and rs2059807 is one of these SNP located in the "hot spot". A haplotype constructed with four SNPs in this region was demonstrated to be associated with PCOS [20], indicating that this might be a PCOS susceptibility loci. Unfortunately, no pooled Odds Ratio (OR) could be calculated due to the failure to obtain original data from the groups conducted the studies. Nevertheless, rs2059807 should be considered a candidate risk factor for genetic predisposition of PCOS observed by some authors [26].

The present study observed significant difference in allele and genotype frequency of INSR gene in PCOS cases the control group in Indian women. It was observed that G/G allele frequency is significantly increased in SNP rs2059806 of INSR gene in PCOS patients when compared to non PCOS control subjects. Results of the present study have clearly shown that A/A genotypes having a less frequency in PCOS patients than controls. Regarding G/G genotypes the opposite trend was observed. The A allele may have a protective role in PCOS. The odds ratio of GG genotype and G allele was more than 1, which suggests that G allele has a role in the causation of developing PCOS.

Limitation(s)

The sample size was limited. It was a single hospital-based study. Biomarkers like adiponectin or measurement of IR were not studied. The present study was also limited by the fact that the number of controls taken were less as compared to the cases.

CONCLUSION(S)

Regarding genotype frequencies of INSR gene, rs2059806 (exon 8, A/G), significant difference in A/A and G/G genotypes were observed between controls and PCOS female whereas regarding allele frequencies A allele was significantly decreased in PCOS female than controls. Polymorphisms of INSR gene, rs2059806 (exon 8, A/G) may be associated with PCOS female. A larger study with further investigations is needed to clarify the impact of this specific genotype on the risk of PCOS.

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